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Conservation Physiology of the Plethodontid Salamanders *Eurycea nana* and *E. sosorum*: Response to Declining Dissolved Oxygen

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Eurycea sosorum* and *E. nana* are plethodontid salamanders endemic to several karst springs in central Texas. Landscapes around these habitats are increasingly urbanized. At the Barton Springs complex, where *E. sosorum* occurs, average dissolved oxygen (DO) in the main flow is approximately 6.5 mg L^{-1}. However, DO is quite variable, ranging between 2.4 and 10 mg O_2 L^{-1}, and recent data suggest a positive relationship between DO and spring discharge in Barton Springs Pool, though this relationship may not be as strong under extreme low-flow conditions. Here we examine sensitivity of a surrogate species, *E. nana*, to experimental variation in oxygen availability (DO); due to limited availability of *E. sosorum*, they were examined in only a subset of experiments. A suite of traits was measured on adults: spontaneous activity, metabolic rate, and mortality during 28 days of exposure. A separate experiment examined growth of juveniles across levels of DO during 60 days of exposure. Levels of DO below 3.4 mg O_2 L^{-1} appeared to pose a grave threat to salamander survival over a 28-day study, whereas DO above 4.5 mg O_2 L^{-1} gave no observable effects in any experiment. Between these values is a critical range in which salamanders became progressively compromised. An ambient water quality criterion for DO in lentic systems (5 mg O_2 L^{-1}, 24 hour minimum) appears adequate to protect *Eurycea*.

GLOBAL amphibian declines over the past half century (Houlahan et al., 2000) appear to have stemmed from factors associated with climate change, including increased UV-B exposure, changes in precipitation patterns, and outbreaks of pathogens (Kiesecker et al., 2001). At local scales, declines also stem from habitat degradation or destruction (Blaustein et al., 1994) related to watershed urbanization (Wang et al., 2001; Price et al., 2006; Miller et al., 2007). Because urban land use influences many aspects of streams—flow regime, channel morphology, water quality, and biological community composition (Wang et al., 2001)—it is difficult to identify specific factors, or interactions of factors, that adversely affect populations. But doing so is important: although urbanization may be inevitable, understanding relative risk associated with various stressors will support better conservation decision-making.

Here we focus on dissolved oxygen (DO), which is known to vary spatially and temporally in aquatic systems (Wetzel and Likens, 2000). The U.S. Environmental Protection Agency has established national ambient water quality criteria for DO that are intended to protect aquatic life in surface waters. In the central Texas karst system at the Barton Springs complex, DO in the main spring has been measured irregularly since 1969. Since then, mean DO has been approximately 6.5 mg L^{-1} (Turner, 2004), with individual measurements ranging between 2.4 and 10 mg L^{-1} (for comparison, air-saturated DO at spring temperature, 20°C, is about 8.5 mg L^{-1}). Moreover, data since 2003 indicate a positive relationship between DO and spring discharge (Turner, 2004). These data suggest that low spring flows, which could stem from either droughts or higher levels of pumping from the aquifer, may subject salamanders to lower DO. Whether the current water quality criteria for DO in surface waters are appropriate for protecting salamanders in spring-fed ecosystems is unknown.

For salamanders, adequate DO is important for all life stages (Hillman and Withers, 1979). Hypoxia can retard embryonic development (Mills and Barnhart, 1999), slow or arrest juvenile growth (Werner and Glennemeier, 1999; Stevens et al., 2006), and depress adult oxygen consumption (Booth and Feder, 1991; Crowder et al., 1998; Sheafor et al., 2000). Identifying problematic levels of DO is difficult, however, because effects vary by species, stage, and physiological circumstance. For example, Withers (1980) showed that O_2 consumption (in air) by resting *Plethodon* spp. was unaffected by ambient PO_2 down to approximately 5 kPa. By contrast, exercised salamanders, forced to escape repeatedly, were much more sensitive to ambient PO_2, with rapid declines in O_2 consumption below 14 kPa. In some circumstances, negative effects of hypoxia may be mitigated by physiology and behavior. Known responses include increases in egg capsule conductance (Mills et al., 2001), precocious hatching (Petranka et al., 1982), increases in heart rate and buccal pumping (Sheafor et al., 2000), behavioral hypothermia (Tattersall and Boutilier, 1997), gill hypertrophy and increases in gill perfusion (Bond, 1960), and frequent excursions to the water–air interface for air or 'bobbing' (Wassersug and Seibert, 1975; Crowder et al., 1998).

Eurycea nana and *E. sosorum* are obligately aquatic neotenes, with gills retained throughout adulthood (perennibranchiate). Oxygen uptake must therefore occur across the skin or the gills; the dominant route is unknown. Booth and Feder (1991) showed that amphibians using cutaneous respiration in water, including *E. bislineata*, can develop steep oxygen gradients across boundary layers adjacent to the skin; even when ambient DO was high (>8 mg L^{-1}), DO at the skin surface usually was 1–2 mg L^{-1}. In *E. sosorum* and *E. nana*, boundary layers near the skin may be minimized by other factors, including small body size (<1 g) and association with rapidly flowing, well-oxygenated spring flows (Sweet, 1982).

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Here we examine sensitivity of juvenile and adult *E. nana* and *E. sosorum* to experimental variation in oxygen availability (DO). Using adult salamanders, we imposed short- to long-term variation in ambient DO and quantified spontaneous activity, metabolic rates, and mortality. For juvenile salamanders, we measured growth rates during 60 days of exposure to different levels of oxygen. This data set provides the most complete multi-stage description of oxygen's effects for any salamander and suggests levels of DO below which physiology, and likely fitness, is compromised. We subsequently performed a probabilistic ecological hazard assessment (PEHA) to relate salamander response thresholds to DO measurements in spring habitats.

MATERIALS AND METHODS

Animals.—Experiments were carried out between November of 2005 to December of 2006. Adult *Eurycea nana* (SVL 22.1–35.1 mm, mean 27.9 mm; Tupa and Davis, 1976) were collected by hand from rocky substrates below the Spring Lake dam (San Marcos, Texas), placed in aerated coolers, returned to Austin, and separated into four ten-gallon holding aquaria. We collected 20 adult *Eurycea sosorum* (SVL 22.9–30.2 mm, mean 26.1 mm) from Eliza Spring during a single collecting trip. Salamanders were collected with the cooperation and supervision of the City of Austin using the same techniques as those described for *E. nana*.

Salamanders were held in ten-gallon aquaria filled with Eliza Spring water. Each aquarium had multiple pieces of pre-soaked PVC tubing for cover, gravel collected from below the Spring Lake dam, an air stone delivering room air, and a filter unit (AquaClear, with mechanical, chemical, and biological filtering capability, 400 liters h^{-1}). We also controlled water pH using a pH-stat system (Milwaukee Instruments model SMS122, Rocky Mount, NC), which measured pH continuously and, whenever it rose above 7.6, injected CO_2 until pH fell below the set point. pH regulated in this way was quite stable, varying between 7.3–7.8 over the course of 15–20 min. Salamanders were kept on a 13L:11D light cycle and fed bloodworms every day (Hikari, with multivitamins added, approximately two bloodworms per salamander). *Eurycea nana* were used in all experiments; *E. sosorum* were used only in measurements of short-term metabolic rates.

Water collection.—Water was collected from Eliza Spring, part of the Barton Springs complex (includes also Eliza Spring, Upper Barton, and Old Mill) that supports the highest density of *E. sosorum* in the wild (pH 7.1–7.5, conductivity about 600 μS cm^{-1}, temperature = 20°C). Water was pumped into food-grade trashcans, transported to the University of Texas campus, and filtered through 0.45-μm PTFE membranes (Pall Life Sciences, TF-450) into two 1,136-liter food-grade polyethylene holding tanks. All holding containers were presoaked with tap water for one week and allowed to air dry before use. Stored Eliza water was aerated continuously with room air.

Spontaneous activity.—Spontaneous activity of *E. nana* (n = 8) was recorded using a modification of the infrared method of Sheafor et al. (2000). Salamanders were confined individually to custom-built, flow-through glass chambers (1.5 × 9 cm), with water driven through the chambers by small gear pumps (Micropump, Vancouver, WA) at 1 cm s^{-1}. Water was recirculated past salamanders from a reservoir, a design that facilitated easy modification of water characteristics (see below). The entire apparatus, including the reservoir, was held underwater in a temperature-controlled water bath (maintained at 20°C). Salamander activity was measured using AD-1 infrared activity detectors (Sable Systems, Las Vegas, NV) with LED emitters and detectors on 70-cm long wires, so that they could be placed directly into the water around the glass chambers. Output voltages from the detectors were sampled once per second onto a computer running Expedata software (Sable Systems, version 2.33).

Individual salamanders were put into chambers, allowed to acclimate for four hours in Eliza Spring water (approximately 660 μS cm^{-1}), then subjected to DO ramp from 8.9 mg O_2 L^{-1} down to 1.3 mg O_2 L^{-1} over 2.5 hours and back up to 8.9 mg O_2 L^{-1} over the subsequent 2.5 hours. Desired levels of DO were obtained by mixing pure O_2, N_2, and CO_2 and bubbling the resulting stream directly into the water reservoir. Gas flow rates were controlled by mass flow controllers (all by Unit Instruments, Milpitas, CA, models UFC-1100 or 1101A; O_2: 0–1 slm or 0–500 sccm; N_2: 0–1 slm or 0–500 sccm; CO_2: 0–10 sccm), which were themselves controlled by a separate electronics package (MFC-4, Sable Systems, Las Vegas, NV). Total flows were approximately 500 ml min^{-1}, and CO_2 flows were adjusted to give a pH of approximately 7.5. Conductivity, pH, and DO were measured continuously with a YSI 556 handheld multiparameter instrument, which was calibrated regularly against standards.

Activity data were analyzed using log survivorship analysis (Slater and Lester, 1982) implemented in S-Plus (v. 6.1). First, raw voltage traces were filtered so that each logged value was classified either as 'no activity' (0) or 'activity' (1). We did this, rather than using raw voltages directly, because there is no linear relationship between magnitude of voltage spike and instantaneous degree of activity (advice from Sable Systems). Individual voltage measurements were considered 'no activity' if they were less than five standard deviations from the mean background noise and 'activity' otherwise. Second, we calculated intervals (N) between every sequential activity event, which were then plotted (as log N) on a histogram. In data traces containing distinct bouts of activity, the log plots show a characteristic concave shape, arising from two different event timings. Within bouts, there is a high probability of subsequent activity (short intervals), and thus at the left side of the graph the slope is steep (corresponding to a high probability of subsequent activity). The shallower part of the trace, to the right, corresponds to between-bout times—i.e., the slope is shallow because the probability of a subsequent event is low.

Historically, the 'bout criterion'—the time distinguishing within bout from between bout intervals—has been identified by eye as the point at which the slope changes most rapidly. However, several authors argue for more quantitative methods of estimation. We used the method of Slater and Lester (1982), which they show minimizes the total number of misclassified intervals. They define the optimal bout criterion as

$$t' = \left(\frac{1}{\lambda_W - \lambda_B}\right) \log\left(\frac{\lambda_W N_W}{\lambda_B N_B}\right),$$

where λ_W and λ_B are slopes of the within- and between-bout parts of the log survivorship graph, N_w is number of intervals in the within-bout section, and N_B is number of intervals in

the between-bout section. The four parameters were estimated for each individual salamander by fitting a double exponential equation to the log survivorship plot, using a non-linear least squares fitting function in S-Plus. Once the bout criterion was identified for each salamander, its activity vector was filtered again to identify regions that were either within activity bouts or between activity bouts.

Responses were modeled with logistic regression, which is appropriate with binary response variables (e.g., active vs. not active). We used both probit and logit links. Fitted coefficients were used to calculate IC_{50}, the level of DO giving activity half the time, as

$$IC_{50} = -a/b,$$

where a is the fitted intercept and b the coefficient for DO. The eight separate estimates of IC_{50} (one per salamander) were then used to calculate mean IC_{50} with 95% CI.

Salamander metabolic rates.—To estimate critical levels of oxygen that cause changes in metabolic rate (Booth and Feder, 1991), we measured metabolic rates of *E. nana* (n = 15) and *E. sosorum* (n = 14) over ramped levels of DO. Oxygen consumption was measured using a semi-closed system. In each metabolic chamber, a perforated nylon insert protected the salamander from a stir bar. A second nylon insert was milled with three ports, one for a mini Clark-style oxygen electrode (model 730, Diamond General, Ann Arbor, MI), and one each for water inlet and outlet (1/8 inch stainless steel). Fits on the stainless steel tubing were tight enough that no additional sealants were used; electrodes were sealed with silicone. The three-port insert was sealed to the glass beaker (100 ml volume) by an o-ring (Buna-N).

Accurate measures of metabolic rate in aquatic systems depends on controlling or measuring several characteristics of the water, including volume, mixing, and biological activity. Water volumes in chambers were measured gravimetrically (47–64 ml). Stir bar rotation was set to mix chamber water thoroughly within 10 s (measured in preliminary experiments using dye dispersal), and the ports allowed us to flush chambers gently while salamanders were in place. When chambers were closed (no flushing), changes in oxygen were due only to biological activity. Extensive testing showed, first, that chambers were essentially leak-free; and, second, biological oxygen consumption by non-salamander sources (e.g., bacteria) were minimal, as introduction of air-saturated water gave stable, air-saturated electrode readings for several hours. To ensure that this was so in every experiment, we always included one or more blank chambers.

The mini electrodes were connected to a picoammeter (Microsensor, Diamond General) via a 10-channel electrode multiplexer (Diamond General, model 1090A), which allowed us to run up to eight salamander and two blank chambers during a single run. Signals from the picoammeter were logged onto a computer via an A/D converter (Sable Systems, UI2, Las Vegas, NV). Electrode membranes (polyethylene, 1 mil thick) were replaced regularly.

To reduce bacterial growth, all chamber parts were washed thoroughly. Electrodes were calibrated at temperature using N_2-purged and air-saturated water. Salamanders were weighed (Mettler Toledo analytical balance, ± 1 mg) and photographed through a stereo-zoom microscope (Nikon SMZ1500 with DS-5M camera) for later analysis of SVL, then placed one to a chamber (up to eight salamanders with two blank chambers) filled with Eliza Spring water (conductivity approximately 680 μS cm^{-1}). Chambers were submerged in a temperature-controlled water bath set to 20°C. Salamanders were given 45 minutes to acclimate, and then each chamber was flushed with five volumes (250 ml) of air-bubbled Eliza Spring water. Using the electrode multiplexer, we then manually stepped through electrodes, measuring O_2 levels in each chamber for 1–2 min. Each chamber was sampled generally five times in 45–60 min, during which time oxygen content fell from air-saturated to a minimum of 80% of air saturation (approximately 7.4 mg O_2 L^{-1}). Subsequently, each chamber was flushed with five volumes of water at a lower level of DO (equilibrated to gas streams generated by mass-flow controllers, as described above).

We used non-linear mixed-effects models, implemented in S-Plus v. 6.1 (Insightful Corporation, Seattle, WA), to examine relationships between DO and metabolic rate. Visual inspection of the data suggested that metabolic rates fell at lower levels of DO. We therefore chose to fit the 'Biochemical Oxygen Demand' model in Bates and Watts (1988),

$$y(x) = \phi_1[1 - \exp(-\exp(\phi_2)x],$$

where y is metabolic rate, x is level of DO, ϕ_1 is the asymptote (in our case, the asymptotic metabolic rate, units mg O_2 hr^{-1}), and ϕ_2 describes how sharply the curve transitions from zero to the asymptote. From fitted values of ϕ_2, the $metIC_{50}$ (the DO giving a 50% reduction in metabolic rate, units mg O_2 hr^{-1}) can be calculated as

$$metIC_{50} = \log 2/\exp(\phi_2).$$

We followed the iterative strategy of Pinheiro and Bates (2000:appendix C.3) for fitting such models in S-Plus, using the function SSasympOrig.

28-day oxygen-toxicity test.—To assess long-term lethal levels of DO, we measured mortality of 60 adult *E. nana* in a 28-d oxygen toxicity test (where low oxygen was the stressor). Salamanders were housed individually in 2-L aquaria, each equipped with an air stone inside a hydraulic lift tube to drive water circulation. Oxygen levels were maintained by bubbling air from the box head spaces into salamander-containing aquaria. Head spaces in the upper chambers were regulated by a multichannel oxygen regulator (ROXY-8, Sable Systems, Las Vegas, NV). To maintain aquarium temperature, the lower halves of the chambers were plumbed for continual recirculation of chilled water (20°C). Aquarium pH was controlled between 7.0 and 8.0 using the pH-stat system described above.

Individual aquaria were arranged three to a Plexiglas chamber (Fig. 1). Plexiglas chambers in the same oxygen treatment were connected via gas lines, with gas flow between them driven by small fans. Twelve salamanders (pseudo-replicates) were randomly assigned to one of five nominal DO exposure treatments, 1.3, 2.4, 3.6, 4.6, and 7.5 mg/L (see Table 1 for measured values), in individual aquaria. Three aquaria were randomly assigned to a given Plexiglas chamber (replicate) providing an experimental design with five treatments and four replicates (Plexiglas chambers) with three pseudo-replicates per replicate (aquaria). Pseudo-replicates were averaged per replicate to provide four values per treatment. During the course of the experiment, there was some mortality from salamander

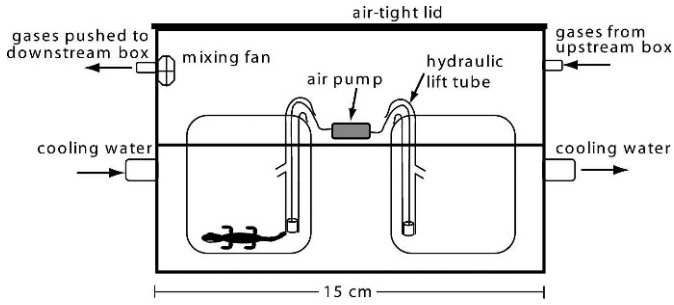

Fig. 1. Experimental set-up for the 28-day oxygen toxicity experiments. Each of 20 controlled atmosphere boxes held three aquaria (one salamander per aquarium); only two aquaria are shown in the figure.

escapes not related to DO level. A total of six escapes and one fungal contaminated salamander resulted in an unbalanced design, with $n = 10$ salamanders in treatments with DO = 3.6 and 4.6 mg/L and $n = 9$ in the DO = 7.5 mg/L.

60-day juvenile growth experiment.—Juvenile salamanders were obtained from the captive breeding program for *Eurycea nana* at the San Marcos (TX) National Fish Hatchery. Juveniles were placed in the same set up as described in the 28-d oxygen toxicity experiment, but DO treatments were set to be non-lethal (see Table 1 for measured values). Juveniles were maintained under these conditions for 60 d. During that time, we weighed and measured snout to vent length (SVL) of each salamander approximately every five days. Juveniles were weighed to the nearest 0.01 mg on a Sartorius MC-5 microbalance. To minimize errors from adherent water and evaporation, salamanders were gently blotted with a dry tissue before being transferred to a weigh boat. Snout–vent length was measured from calibrated digital images. Due to limited availability of juveniles from the captive breeding program, we were able to place only five salamanders into each treatment at the beginning of the experiment.

Toxicity data analysis.—For the 60 d juvenile growth study specific growth rate (G_W), defined as the rate of change of the logarithm of weight through time, was calculated as

$$G_W = 100 \cdot (\ln (W_{final}/W_{initial})/t),$$

where $W_{initial}$ is salamander weight at the beginning of the experiment, W_{final} is weight at the end, and t is time (days). Lowest observable adverse effect level (LOAEL) and no observed adverse effect level (NOAEL) thresholds were determined using Bonferroni's *post hoc* test (USEPA, 2002).

Data for both the 28 d lethality study and the 60 d juvenile growth study were modeled using the linear and non-linear equations outlined in Table 2 (Brain and Solomon, 2007). Model fit was based on the coefficient of determination and the *P*-value for each associated ANOVA. Each model employs an iterative process by fitting parameters simultaneously. If the convergence criteria (approach to stable parameter values) are not met in a specified number of iterations, the model cannot be fit. Based on the variability and distribution of the data, tolerance criteria may not be met for a given model; thus, multiple models were tested. To optimize the fitting process, we adjusted number of iterations, step sizes, and thresholds of tolerance. Effective (60 d juvenile growth study) or lethal (28 d lethality study) concentrations required to inhibit or kill x percent of the organisms (EC_x or LC_x) were calculated, with x set to 5, 10, 25, and 50.

Table 1. Measured Oxygen Levels in the 28-d (Adult Toxicity) and 60-d (Juvenile Growth) Experiments.

	28-day		60-day	
Treatment	mean DO (mg L^{-1})	Std. err.	mean DO (mg L^{-1})	Std. err.
1	1.7	0.32	4.4	0.28
2	2.8	0.34	5.0	0.36
3	3.1	0.28	5.3	0.18
4	4.6	0.13	6.0	0.31
5	7.3	0.10	8.0	0.52

Dissolved oxygen distribution.—Data for Barton Springs DO were acquired from the City of Austin, which was originally obtained from the U.S. Geological Survey (Chris Herrington, pers. comm.). This dataset, containing 517 DO observations taken between November 1969 and April 2009, was plotted according to published methods (Solomon and Takacs, 2002) as a cumulative frequency distribution, with probability on the y-axis and $\log_{10}$ DO on the x-axis (Solomon et al., 2000). Plotting positions (j) were expressed as percentages and calculated from the Weibull formula

$$j = 100 \cdot i/(n+1),$$

where i is the rank and n is the total number of data points in the data set. Linear regressions were performed on the transformed data using SigmaPlot 2000 (SPSS, Chicago, IL. http://www.sigmaplot.com). This approach is conceptually similar to an approach recently proposed for anoxia thresholds of benthic marine invertebrates (Vaquer-Sunyer and Duarte, 2008).

Toxicity threshold calculations.—Low centiles of 1% and 5% from the DO distribution were considered potentially appropriate thresholds of exposure and used as Toxicological Benchmark Concentrations (TBCs; Hanson and Solomon, 2002) for this initial assessment. The first centile represents a conservative lower bound of the probabilistic distribution, whereas the fifth centile is analogous to the HC_5 (5^{th} centile hazardous concentration; concentration affecting 5% of species and therefore protective of 95% of species) derived from a species sensitivity distribution of toxicity values (Wagner and Lokke, 1991; Aldenberg and Slob, 1993; Sijm et al., 2002). Hence, based on the DO exposure distribution(s), 99 and 95% of DO concentrations are expected to fall above these thresholds, respectively.

Probabilistic ecological hazard assessment (PEHA).—We performed a PEHA that used the observed DO distribution from Barton Springs, and the LC_5, LC_{10}, LC_{25}, LC_{50}, and 60 d NOAEL and LOAEL thresholds calculated for the 60-d chronic study. A PEHA indicates the likelihood that a DO value will be encountered in Barton Springs that is below the indicated threshold for *Eurycea nana*. This calculation was done by modifying equations from Solomon et al. (2000) as outlined in Brain et al. (2006). We substituted a single threshold value for percentage-based exposure values using Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA) as

$$P_x = NORMDIST(m_{tox} \cdot \log_{10}(x) + b_{tox}),$$

Table 2. Equations Used to Fit the Concentration–Responses of *Eurycea nana* Exposed to Varying Dissolved Oxygen Levels. The variable LC_x is the calculated effective concentration at which proportion p of the endpoint is affected, and x is the actual concentration (mg L^{-1}), y is the response or change from control of the endpoint modeled, and a, b, and y_0 are constants.

Regression	Equation	Modeling type
Linear	$y = a+((ap)/LC_x)x)$	Increase
Four parameter logistic	$y = y_0+a/(1+(x/LC_x)^b)((a/(1-p)(y_0+a)-y_0)-1)$	Decrease
Four parameter logistic	$y = y_0+a/(1+(x/LC_x)^b)((a/(1+p)(y_0+a)-y_0)-1)$	Increase
Three parameter logistic	$y = a\ (1+(p/(1-p)(x/LC_x)^b))$	Decrease

where x is the threshold exposure value, P_x is the probability of encountering a DO value below the designated threshold (x), NORMDIST returns the standard normal cumulative distribution function, and m_{tox} and b_{tox} are the slope and intercept, respectively, of the probit/log transformed regression line of the exposure data.

RESULTS

Spontaneous activity.—All eight *E. nana* in the DO ramp had discernable breakpoints that identified activity bouts (see Fig. 2). Mean bout criterion was 1.60 minutes (range 0.82–2.56).

Salamanders had a clear onset of activity as DO dropped to between 2.7 and 5.5 mg O_2 L^{-1} (Fig. 3A). During the ramp back up, activity ceased at a lower level of DO, approximately 1.8–4.1 mg O_2 L^{-1}. Figure 3B summarizes salamander activity during the experiment. For each salamander, we fitted a logistic regression model separately to rising and falling parts of its activity curve, estimated each IC_{50}, then calculated means and 95% CI across the eight salamanders. Probit and logit links gave virtually identical results, so we present averages of the two techniques. For the rising part of the activity curve (declining DO), the DO at which 50% of salamanders became active was 4.54 mg O_2 L^{-1} (95% CI 4.02–5.06). For the falling part of the activity curve (increasing DO), the DO at which 50% of salamanders became inactive was 3.12 mg O_2 L^{-1} (95% CI 2.39–3.86). Changes in activity thus exhibited some hysteresis.

Salamander metabolic rates.—Metabolic data were quite variable, both within and between salamanders. Nevertheless, the two species had similar average metabolic rates, and the metabolic rates clearly declined at low levels of DO (Fig. 4A, B), especially below 3 mg O_2 L^{-1}.

Estimates of $metIC_{50}$ were obtained using Eq. 4. For *E. nana* we estimate $metIC_{50}$ = 1.31 mg O_2 L^{-1} and for *E. sosorum* $metIC_{50}$ = 1.62 mg O_2 L^{-1} (Table 3). The confidence intervals for both parameters, ϕ_1 and ϕ_2, were broadly overlapping, so we consider species' responses to DO to be statistically indistinguishable. Estimated values for ϕ_1 (metabolic rate under non-limiting oxygen conditions) were 0.052 and 0.043 mg O_2 hr^{-1} for *E. nana* and *E. sosorum*, respectively.

28-day oxygen-toxicity test.—There was a clear logistic relationship between DO and percent mortality (Fig. 5), with mortality falling from high to low between approximately 2 and 4 mg O_2 L^{-1}. Salamander mortality related to DO occurred in the lowest three treatments (1.3, 2.4, and 3.6 mg/L), and all mortality that occurred in the two lowest DO treatments happened within 48 hours of initiating the experiment. No DO related mortalities were observed in either of the two highest treatments (4.6 and 7.5 mg/L). LC_5, LC_{10}, LC_{25}, and LC_{50} estimates were calculated for adult mortality data (Table 4) using a three parameter logistic model (r^2 of 0.93; Fig. 5) these values were considered thresholds of response for *E. nana* exposed to varying DO concentrations.

60-day juvenile growth experiment.—Although juveniles in the lowest DO (4.4 mg O_2 L^{-1}) had growth rates that were approximately 30% lower than control salamanders (Table 5), the differences were not significant when analyzed by linear mixed-effects models, perhaps because both the sample sizes and the DO range were small (n = 4 or 5 per treatment). Using a toxicological approach, we determined that the specific growth rate NOAEL was 4.4 mg O_2 L^{-1} ($P > 0.05$; Table 4), the lowest DO examined. Therefore, a lowest observable adverse effect level (LOAEL) was not determined. However, had growth rates in 4.4 mg O_2 L^{-1} been just slightly lower, they would have been significantly different from controls ($P < 0.05$) based on minimum significant difference values. This indicates that the growth NOAEL of 4.4 mg O_2 L^{-1} closely approached a LOAEL for juvenile *E. nana* over a 60-d period. A similar analysis using absolute growth rate for each salamander—calculated as the slope of its mass over time—gave similar results (no significant effect of DO at $P < 0.05$).

Probabilistic ecological hazard assessment.—The linear regression equations generated from the probability and $\log_{10}$ transformed DO data for Barton Springs, Eliza Spring, and Old Mill sampling locations (Fig. 6) were $y = 12.5x-9.8$, $y = 13.2x-10.1$, and $y = 6.1x-4.5$, respectively. The probabilities of exceedance (the probability of encountering a DO value below the specified biological threshold; LC_x or NOAEL), based on these DO distributions at the three sampling locations, and calculated using the LC_x estimates generated from the 28-d study with adult *E. nana* thresholds (mortality) and a 60-day NOAEL (specific growth rate), are summarized in Table 4. The exceedance values for Barton Springs and Eliza Spring were similar; however, Old Mill had substantially higher exceedance estimates owing to a flatter slope and lower measured DO values. However, the correlation coefficient (r^2) for the regression line fitted to the Old Mill data was also lower (0.65) than those for Barton Springs and Eliza Spring (0.97 and 0.96, respectively). In addition, inspection of the data (Fig. 6) indicates that the flow–DO relationship at Old Mill was not log-linear. Nonetheless, there were many low DO values, potentially related to low spring flows, compared to the other two sites, causing a shift in the curve and resulting in loss of linearity. Consequently, greater confidence is placed on estimates generated from Barton Springs and Eliza Spring data.

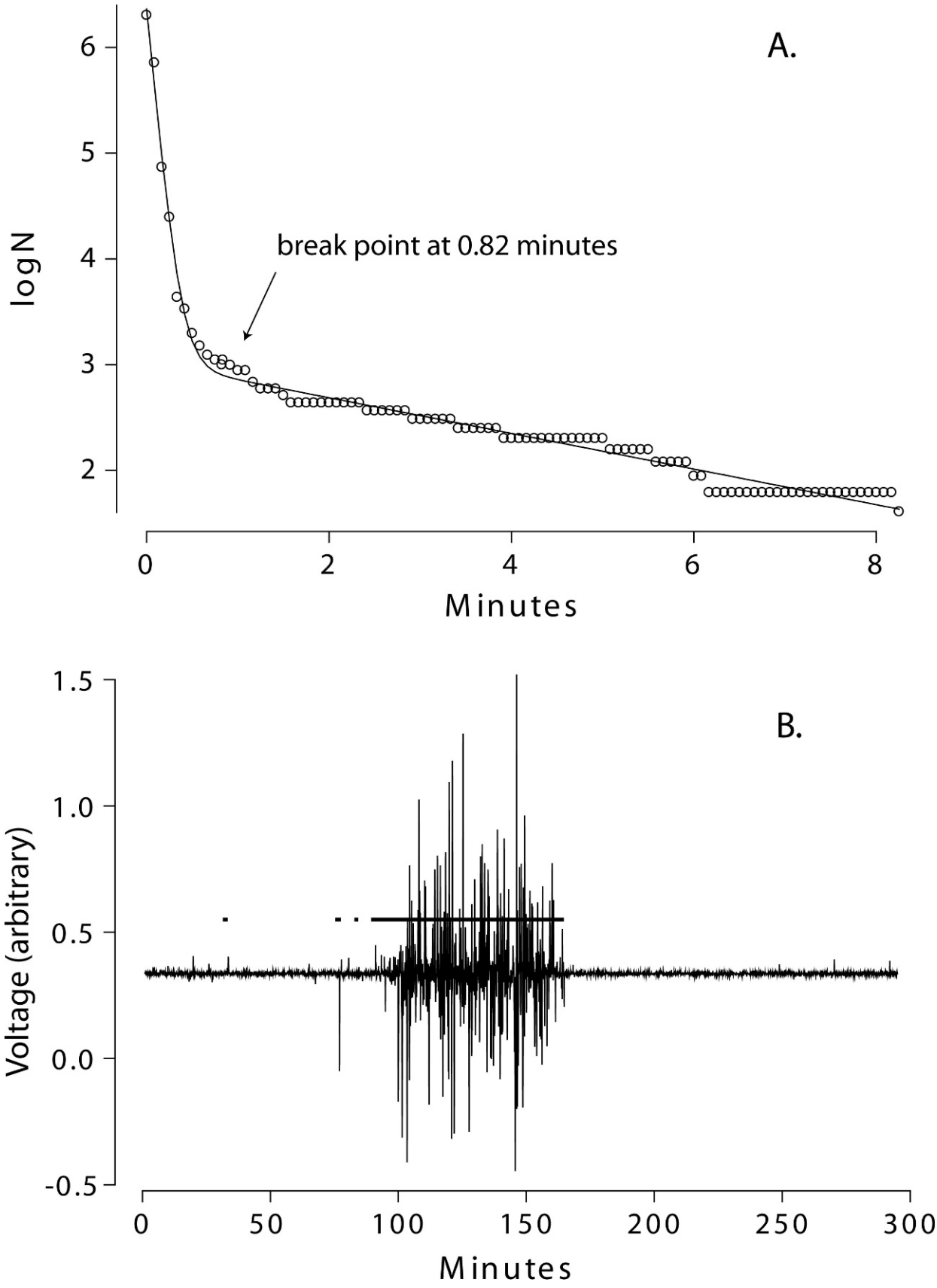

Fig. 2. Example of the log survivorship analysis of activity for one of the salamanders showing (A) the location of the breakpoint at 0.82 min between activity bouts and (B) raw voltage trace from infrared activity meter with activity bouts drawn above according to the breakpoint identified in (A).

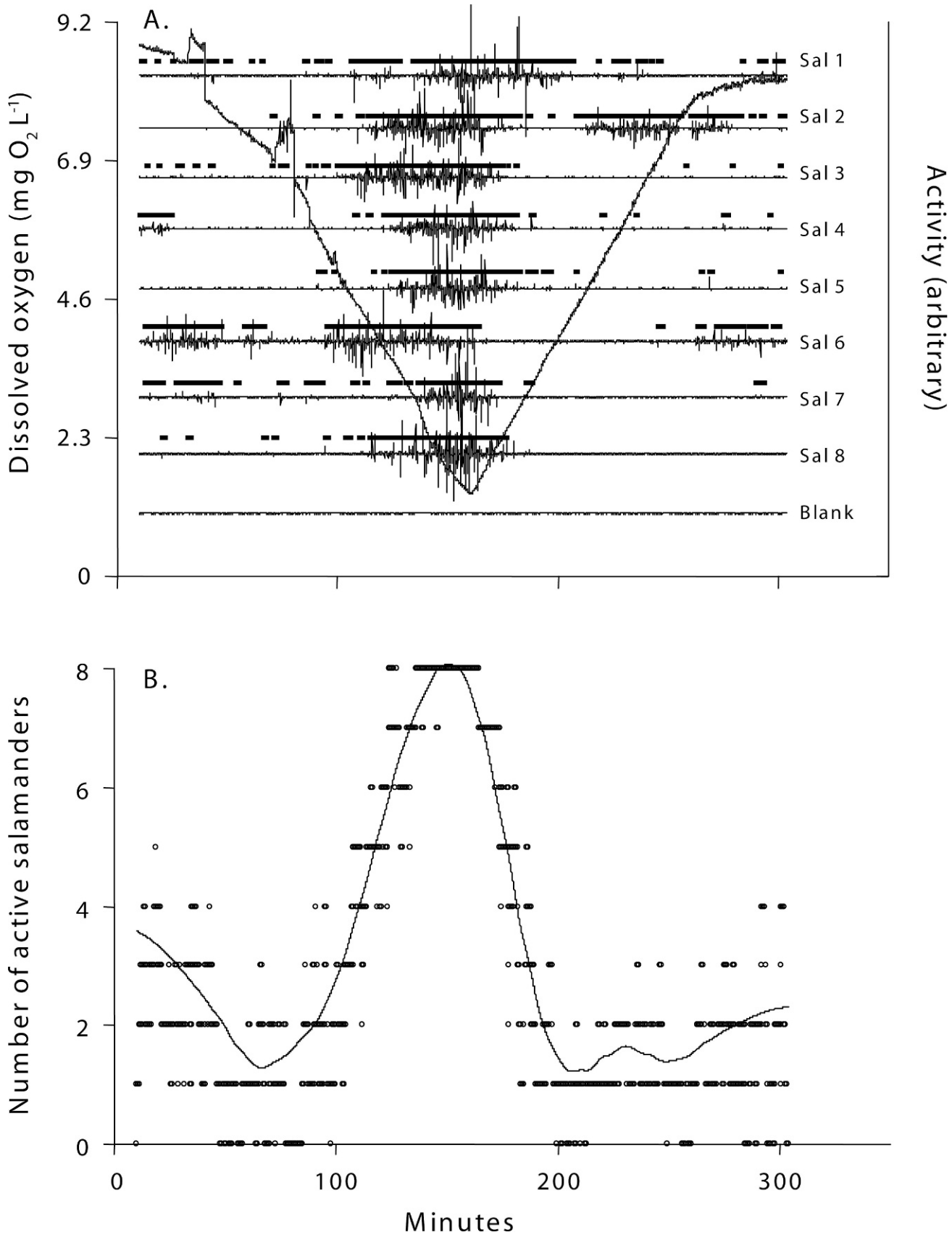

Fig. 3. Spontaneous activity of *Eurycea nana* in response to ramped dissolved oxygen. (A) Raw voltage traces and fitted bouts for each of eight salamanders and a blank chamber superimposed on the trace of dissolved oxygen. (B) Dots are total number of salamanders active (out of eight), and the line is a fitted loess curve (local regression, with smoothing, smoothing parameter = 0.3).

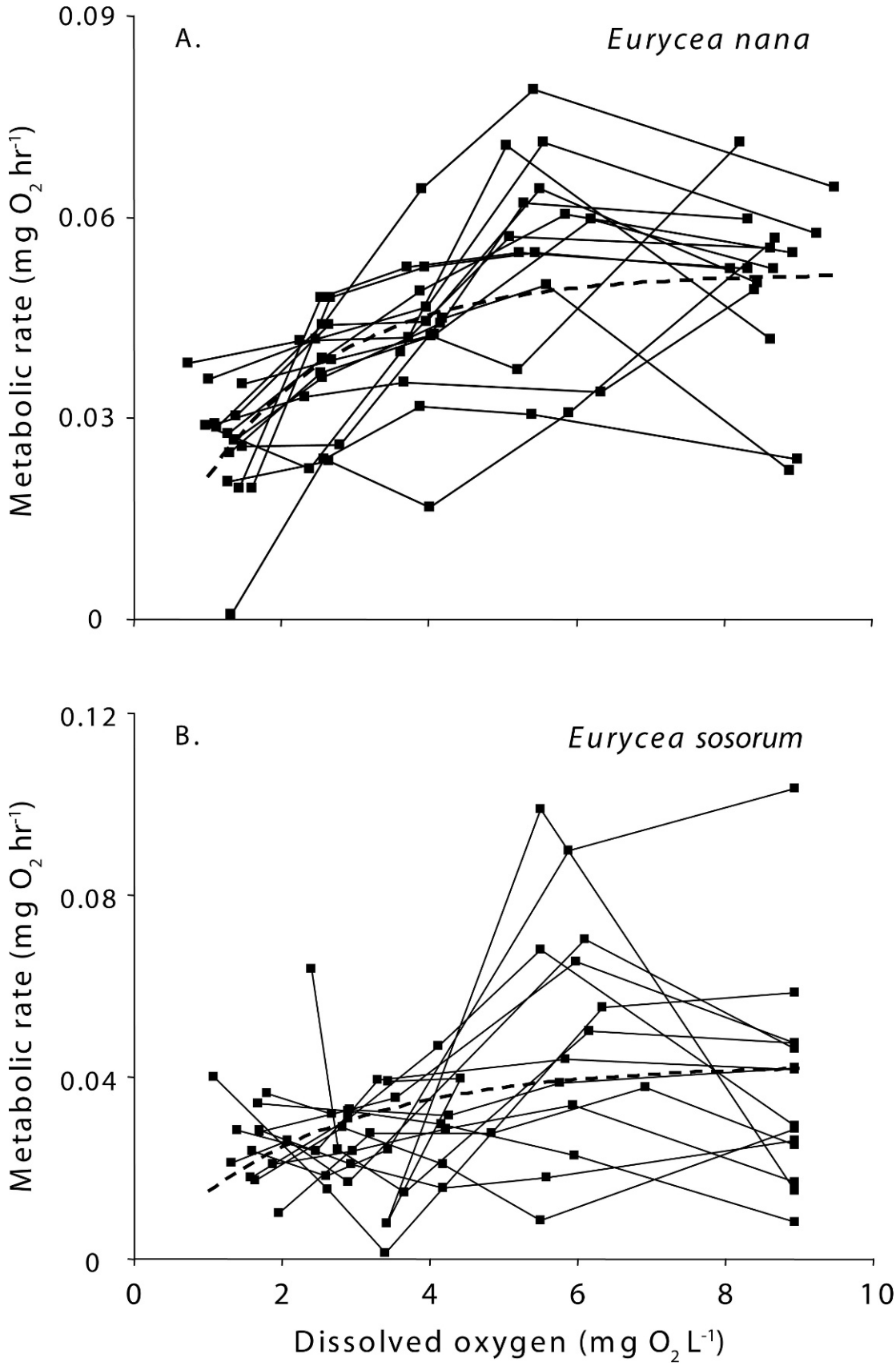

Fig. 4. Metabolic rates of *Eurycea nana* (A) and *E. sosorum* (B) across ramped levels of dissolved oxygen (DO). Lines represent best fits of the Biological Oxygen Demand model (Eq. 3). See Table 3 for summaries of parameter values and statistical significance.

Table 3. Summary of Parameter Values and Statistical Significance from Fitting the Biological Oxygen Demand Model (Eq. 3) to Data on Metabolic Rates as a Function of Dissolved Oxygen Levels (see Fig. 5). $metIC_{50}$ was calculated from Eq. 4.

Species	Parameter	Value	95% CI	num df	den df	*F*	*P*
E. nana	ϕ_1	0.052	0.045 to 0.058	1	59	251.6	<0.0001
	ϕ_2	−0.64	−0.37 to −0.90	1	59	23.5	<0.0001
	$metIC_{50}$	1.31	1.01 to 1.70				
E. sosorum	ϕ_1	0.043	0.032 to 0.053	1	55	85.7	<0.0001
	ϕ_2	−0.85	−1.48 to −0.22	1	55	7.03	0.01
	$metIC_{50}$	1.62	0.86 to 3.04				

As summarized in Table 4, the probability of toxicological threshold exceedances (proportion of DO values below thresholds) for Old Mill ranged from 11% to 38%. For Barton Springs and Eliza Spring the exceedance estimates were similar, ranging from 0.08 to 5.2% and 0.1 to 6.8%, respectively. Based on the DO data from Barton Springs and Eliza Spring there is a 4.5% and 5.8% chance, respectively, that daily DO concentrations will drop below 4.4 mg O_2 L^{-1} (the 60 d NOAEL) that would adversely affect juvenile *E. nana* specific growth rate, a widely accepted parameter linked to population level stress (Suter, 2007). In Old Mill, there is a 28% chance that DO will drop below 4.4 mg O_2 L^{-1} during daily observations

Toxicological Benchmark Concentrations were calculated for low centiles of 1% and 5% based on the DO distributions for Barton Springs at 4 and 4.5 mg O_2 L^{-1}, for Eliza Spring at 3.9 and 4.4 mg O_2 L^{-1}, and for Old Mill at 2.3 and 2.9 mg L^{-1}, respectively. In the absence of more complete data, these values may represent reasonable thresholds of response and indicate that there is ≤1% chance that the DO values will fall below 4, 3.9, and 2.3 mg O_2 L^{-1}, respectively, at Barton Springs, Eliza Spring, and Old Mill, and ≤5% chance that DO will fall below 4.5, 4.4, and 2.9 mg O_2 L^{-1} at the same locations, respectively. It is important to note that this PEHA is driven by probability of discrete and daily average DO values exceeding toxicity thresholds determined from 28-d adult mortality and 60-d juvenile growth studies. Future efforts are needed to determine probabilities of encountering DO exceedances of such thresholds over sustained time periods corresponding to laboratory DO experiments (e.g., 28, 60 d).

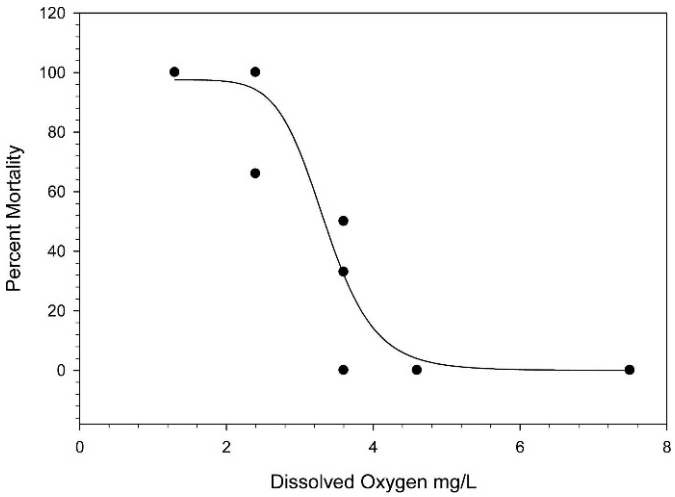

Fig. 5. Percent mortality of *Eurycea nana* exposed to varying dissolved oxygen content.

DISCUSSION

Although species declines stem from multiple factors, more than 70% of endangered organisms are adversely affected by habitat destruction (Pattee et al., 2003). For these species, management decisions often are supported by analyses of ecological hazard or risk (Suter, 2007), with risk assessed in relation to populations. For threatened and endangered species, however, risk may also be assessed in relation to individuals (Suter, 2007), as we have done here. Furthermore, some populations may be imperiled enough that detailed physiological or ecological studies simply cannot be done. Historically, this situation has been approached by studying surrogate species, and sophisticated models are available for analyzing correlations between the responses of surrogates and threatened or endangered species (Raimondo et al., 2007). In this study, we selected *E. nana* as a surrogate because its genetics and life history are similar to those of *E. sosorum* (Chippindale et al., 2000), it occupies similar karst-fed springs in central Texas, and the two species have similar physiologies. Although a lack of even minimal data on *E. sosorum* prevented us from applying formal correlation analyses (Raimondo et al., 2007), our data on *E. nana* provide important, novel insights into how *E. sosorum* is likely to respond to different levels of DO.

Physiology has much to offer conservation, by providing mechanistic insight into links between environmental factors and animal performance (Feder, 1983; Ricklefs and Wikelski, 2002; Helmuth et al., 2005). In turn, understanding performance should allow us to develop prospective views of how animal populations will change in response to stressors and degradation of habitat quality. In practice, establishing strong links between select physiological measures and population processes can be difficult, for two reasons. First, environmental change may affect multiple aspects of performance (e.g., behavior and physiology), and it may be difficult to identify *a priori* which aspects are most important, though the relationship of sensitivities among endpoints is understood for many chemical and physical stressors (Suter, 2007). Second, most animals have complex life cycles (Werner, 1988), and distinct stages can respond to changing environments in different ways.

We analyzed effects on *Eurycea* of an environmental factor, DO, that varies substantially in the habitat of interest (the Barton Springs complex) and that affects other aquatic organisms in profound ways. To assess links between variable DO and salamander population-level processes, we analyzed the effects of DO on fitness-related physiological and behavioral characters (spontaneous activity levels, metabolic rates, survival probabilities, and growth rates) across more than one life stage (juveniles and adults). This approach provides data-rich views of salamander biology,

Table 4. Lethal Concentrations (LC_x) of Oxygen Required to Cause Mortality in 5, 10, 25, and 50% of *Eurycea nana* during 28 Days of Exposure[a] and No Observable Adverse Effect Level (NOAEL) for a 60-Day Exposure. The probability of exceedance for each of the threshold values is provided based on calculations using a probabilistic hazard assessment model (Equation 2) for dissolved oxygen data from Barton Springs, Eliza Springs, and Old Mill sites.

Effect	Type	Regression model	Value (mg L^{-1})	*P*	Probability of exceedance (% of values below threshold)		
					Barton Springs	Eliza Spring	Old Mill
LC_5	28 d	3-parameter logistic	4.5 ± 0.5	<0.0001	5.2	6.8	30
LC_{10}	28 d	3-parameter logistic	4.2 ± 0.3	<0.0001	2.3	3.024	
LC_{25}	28 d	3-parameter logistic	3.7 ± 0.1	<0.0001	0.4	0.4	15
LC_{50}	28 d	3-parameter logistic	3.4 ± 0.2	<0.0001	0.08	0.1	11
NOAEL	60 d	Bonferroni	4.4	>0.05	4.5	5.8	28

while also highlighting further gaps that would have been useful to examine but were not within the scope of the project, for example, how DO affects reproduction, egg development, and hatching.

Effects of DO on salamander activity.—A potentially important response to low DO is mitigation. In most habitats, salamanders will occur across mosaics of high and low DO (or of other factors, such as water flow rate, that affect O_2 availability). Although sensing and responding to such mosaics may be irrelevant at high average DO levels, it surely becomes more important at low DO. In our experiments, salamanders clearly perceived and responded to low (or falling) DO, as the infrared detection system measured onset of activity during falling DO and cessation of activity during subsequent rising DO (Fig. 3).

We interpret activity as having either of two mitigation functions. The more likely is escape from low DO into higher DO areas (though this was not possible for salamanders in our experiments). In the wild, salamanders in local pockets of low-DO water may find higher-DO water nearby (Nolan and Ultsch, 1981). Rigorously assessing this possibility would require measuring the spatial scale of DO variation in natural habitats (Revsbech and Jorgensen, 1986; Dodds, 1991; Kemp and Dodds, 2001). This interpretation is consistent with patterns of salamander presence and absence in the Barton Springs complex. Counts of *E. sosorum* decline in Barton Springs when DO falls below 5 mg O_2 L^{-1} (Turner, 2004). It is likely that salamanders move into the karst system during periods of low DO; however, it is not known whether recolonizing salamanders are the same individuals as those leaving.

A second function of increased activity may be to minimize boundary layers adjacent to skin and gills. Water flow rates in our experiments were, for technical reasons, fairly low (1 cm s^{-1}), likely giving substantial boundary layers. Salamanders may increase oxygen flux to sites of respiratory exchange by disrupting those boundary layers, for example, by bobbing, flicking their heads, or swimming (Wassersug and Seibert, 1975; Crowder et al., 1998).

Table 5. Summary of Growth Rates of Juvenile *Eurycea nana* over 60 Days in Different Dissolved Oxygen Levels.

Treatment	DO (mg L^{-1})	*n*	Growth rate (mg d^{-1})	Std. err.
1	4.44	5	0.15	0.04
2	5.17	4	0.33	0.07
3	5.31	4	0.26	0.03
4	6.35	5	0.24	0.05
5	8.22	4	0.23	0.06

Effects of DO on salamander physiology, survival, and growth.—The three traits, respiration rate, 28-d adult survival probability, and 60-d juvenile growth rate, were differentially sensitive to DO. In particular, $metIC_{50}$ (acute exposure giving 50% depression of oxygen consumption rate) was low. For *E. nana* it was 1.3 mg O_2 L^{-1} and for *E. sosorum* 1.6 mg O_2 L^{-1}. In the 28-d oxygen toxicity test, the LC_{50} (giving 50% reduction in survival) was higher, 3.4 ± 0.2 mg O_2 L^{-1}. This difference may reflect that particular levels of low DO are worse for salamanders the longer their exposure to it. However, in the 60-d juvenile experiment, we observed no significant effects of low DO on growth rate, with the caveat that sample sizes were small and our range of experimental DO levels did not extend below 4.4 mg O_2 L^{-1}. Future studies should assess growth under lower oxygen levels and after acclimation to various DO concentrations.

Linking dissolved oxygen to population persistence.—This study was motivated by a conservation problem: *E. nana* and *E. sosorum* are threatened and endangered, respectively, and exist only in small sets of springs surrounded by urban areas. Water quantity and quality in the springs vary over time, with flow and DO positively correlated for Barton Springs (City of Austin, 1997). Historically, variation in flow has been driven by weather and climate on the Edwards Plateau, the limestone escarpment that is the source of aquifer water feeding the springs. At present, variation in flow likely is influenced also by human water use (Slade et al., 1985; Smith and Hunt, 2004). Pumping appears to increase the likelihood of low water flows and associated low DO. Unfortunately, there are few available observations of DO concentrations at low flows. For example, only 27 observations of flow below 20 c.f.s. were included in the dataset used for a PEHA in this study (Fig. 6), and the mean DO value associated with these low flows is 4.69 (±0.28) mg O_2 L^{-1} for Barton Springs. Further, there were only 35 DO observations for Barton Springs below 4.5 mg O_2 L^{-1} in the available dataset (Fig. 6), and there was no statistically significant ($P > 0.05$) relationship between these low flow and associated DO values. Less information for Eliza Spring and Old Mill precluded similar evaluations here.

Other factors such as nutrients and oxygen-demanding wastes, which are known to influence DO variability and

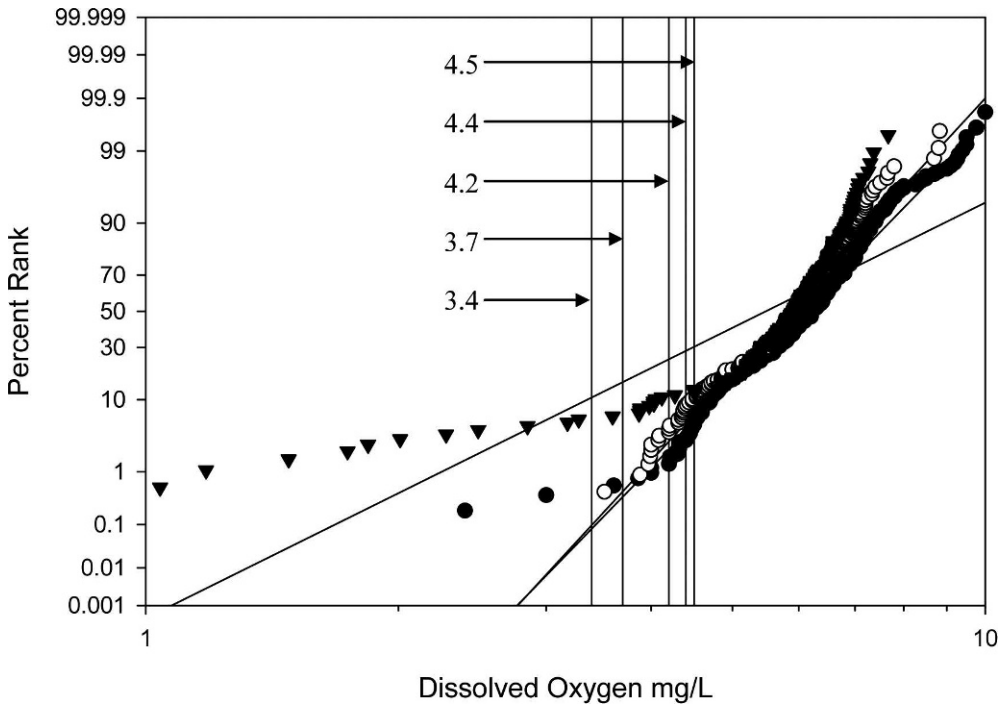

Fig. 6. Percentage rank and log-transformed plot for a distribution of discrete dissolved oxygen measurements for Barton Springs ●, Eliza Spring ○, and Old Mill ▼ locations in central Texas. The corresponding correlation coefficients for the regression lines fitted to each sampling site are 0.97, 0.96, and 0.65, respectively. Vertical reference lines represent the LC_{50} (3.4 mg L^{-1}), LC_{25} (3.7 mg L^{-1}), LC_{10} (4.2 mg L^{-1}), LC_5 (4.5 mg L^{-1}), and NOAEL (4.4 mg L^{-1}), respectively, for 28 d adult mortality and 60 d juvenile specific growth rates of *Eurycea nana* exposed to varying dissolved oxygen concentrations.

daily minima, are targeted by regulatory agencies under the U.S. Clean Water Act to protect aquatic life in inland waters (TCEQ, 2003). A recently developed water quality protection plan for the Barton Springs segment of the Edwards Aquifer identified a number of factors associated with urbanization that may result in water quality stress to endemic salamanders (Naismith Engineering, Inc., 2005). Compared to groundwater withdrawals, the relative contribution of landscape practices and nutrient enrichment on regulation of diel, seasonal, and interannual DO dynamics in habitats of *Eurycea* is not understood, but is likely significant.

A key question is how salamander populations will fare in different levels of DO. The most severe effect would be large-scale mortality of one or more life stages. For example, adult *E. nana* in the 28-d toxicity test had an LC_{50} of 3.4 mg O_2 L^{-1}. Clearly, DO levels $\leq$ 3.4 mg O_2 L^{-1} would constitute a grave threat to populations if conditions persisted for 28 d. The probability of such an event is low (Table 4). However, it is worth also considering less severe conditions, as these have substantially higher probabilities of occurring in the Barton Springs complex: the LC_5 and LC_{10} values are likely to be exceeded with probabilities (percentage of DO values below thresholds) of 5.2% and 2.3%, respectively, over short time intervals (discrete sampling). Certainly, exceedance probabilities will be lower for 28-d periods, but how much lower is unknown. Several additional kinds of data would help resolve this issue: real-time DO diurnal monitoring (e.g., with multiparameter datasondes) in *Eurycea* habitats, more modeling of the probability of long-duration, low-DO events, and the effects on adults of more natural time courses of DO cycling. For the present discussion, an important caveat is that DO toxicity testing was done on adults only. If other stages, eggs or juveniles, are more sensitive (exhibit higher LC_{50}s), higher levels of DO may still constitute a considerable threat. For example, no data are available to evaluate mortality responses of eggs of *Eurycea* to DO. Although eggs are small, which should relieve boundary layer resistance to oxygen flux, they are also immobile and, especially early in development, may have poorly developed systems for coping with oxygen variability.

The converse is to ask: above what level of DO did we observe no statistical change in any of the measured traits? In the growth experiment, there were no observable effects of DO $\geq$ 4.4 mg O_2 L^{-1}, and in the acute experiment there was 10% mortality (LC_{10}; considered equivalent to a NOAEL [TenBrook et al., 2009]) at 4.2 mg O_2 L^{-1}. Metabolic rates appeared only slightly depressed in this range. The spontaneous activity experiment indicated an intermediate sensitivity to DO (IC_{50} of 4.5 mg O_2 L^{-1}).

The DO range between these extremes, of large-scale mortality at 3.4 mg O_2 L^{-1} versus no observable effects above approximately 4.5 mg O_2 L^{-1}, is the location of greatest biological interest. It is likely that populations in the Barton Springs complex would fare increasingly poorly in lower DOs persisting for 28–60 d periods within this range, but how poorly is unknown. Quantitative assessment of these thresholds awaits additional, field-oriented studies.

To relate laboratory stressor–response data to ambient DO values in habitats of *Eurycea*, we performed a PEHA for three spring-fed systems in the Barton Springs Complex: Barton Springs Pool, Eliza Spring, and Old Mill. The PEHA suggests that the fifth centile values of average daily DO (4.5 and 5.8 mg O_2 L^{-1} in Barton Springs Pool and Eliza Spring, respectively) are sufficient to protect juvenile and adult *Eurycea*, as the NOAEL for juvenile growth rates over a 60 d period was 4.4 mg O_2 L^{-1}. However, the likelihood of

exceeding ecologically meaningful DO thresholds is much higher in Old Mill (Table 4). These observations suggest that we need a better understanding of the physical, chemical, and biological factors influencing DO below 4.5 mg O_2 L^{-1} in spring-fed habitats of *Eurycea*, particularly given endangered and threatened species concerns and potential Type II errors (Brosi and Biber, 2009).

In Texas, DO water quality criteria for the protection of aquatic life are prescribed for streams/rivers and reservoirs (lakes) as 24 hr average and absolute minimum concentrations, though water quality criteria for other aquatic habitats are not as well defined or understood (Brooks et al., 2008). For example, Barton Springs Pool is considered an unclassified water body with a high aquatic life use and a DO water quality criterion of 5 mg L^{-1} over a 24 hr period (TCEQ, 2003). Thus, DO water quality criteria for lentic systems (5 mg O_2 L^{-1}, 24 hr average) appear to offer adequate protection to *Eurycea*, though future studies are required to define whether *Eurycea* are protected by absolute 24 hr minimum DO water quality criteria applied to high aquatic life use habitats. In addition, Barton Springs Pool, Eliza Spring, and Old Mill are spring-fed surface waters (neither river nor reservoir) with unique physical features known to influence the production–respiration dynamics of ecosystems and, thus, DO (Forbes et al., 2008). Due to data availability and the scope of the present study, we were unable to fully examine whether river DO water quality criteria protect these threatened and endangered salamanders. Further research is needed on how spatial and temporal variation in DO affects the life history and resiliency of *Eurycea*. Future efforts should determine the influence of urbanization and climate variability on water quality and associated ecological thresholds for *Eurycea*.

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